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Simultaneous Analysis of Glycosylated and Sialylated PSA Reveals Differential Distribution of Glycosylated PSA Isoforms in Prostate Cancer Tissues

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Abstract

Aberrant protein glycosylation has been shown to be associated with disease progression and can be potentially useful as a biomarker if disease-specific glycosylation can be identified. However, high-throughput quantitative analysis of protein glycosylation derived from clinical specimens presents technical challenges due to the typically high complexity of biological samples. In this study, a mass spectrometry-based analytical method was developed to measure different glycosylated forms of glycoproteins from complex biological samples by coupling glycopeptide extraction strategy for specific glycosylation with selected reaction monitoring (SRM). Using this method, we monitored glycosylated and sialylated prostate-specific antigen (PSA) in prostate cancer and non-cancer tissues. Results of this study demonstrated that the relative abundance of glycosylated PSA isoforms were not correlated with total PSA protein levels measured in the same prostate cancer tissue samples by clinical immunoassay. Furthermore, the sialylated PSA was differentially distributed in cancer and non-cancer tissues. These data suggest that differently glycosylated isoforms of glycoproteins can be quantitatively analyzed and may provide unique information for clinically relevant studies.

INTRODUCTION

Glycosylation is one of the most common post-translational modifications of proteins. It has been found to occur in more than half of eukaryotic proteins and is involved in a variety of biological activities^{1–3}. In particular, the importance of differential glycosylation of complex glycans from membrane-bound and extracellular proteins has been demonstrated in clinically relevant studies such as novel biomarker discovery, new drug and therapeutic developments^{4–7}. The biological basis of these observations may be explained by the fact that glycosylated extracellular and membrane bound proteins can serve as receptors on cellular surfaces and perform various structural and functional roles⁸. Thus accurate and high-throughput quantification of protein glycosylation in clinical specimens may provide detailed information on changes correlated with different disease states. These molecular biomarkers could then potentially be used for early disease detection or therapeutic drug development.

Currently, there are two major methods for quantification of protein glycosylation. One relies on the analysis of glycoproteins with specific glycan motifs and is exploited in several

techniques used in glycomic research, such as chemical immobilization of glycopeptides, affinity chromatography, lectin microarray, and lectin-antibody immunoassay^{9–12}. Another quantification method is glycan analysis using MS-based technology^{13–15}. In glycan analysis, glycoproteins from complex samples are separated and concentrated using chromatography or electrophoresis in order to obtain purified glycoproteins. Then, the glycans are released from glycoproteins for structural and quantitative analyses using tandem mass spectrometry. However, the multiple-step sample preparation for glycan analysis reduces quantification accuracy and limits throughput. This presents additional hurdles for analyzing large numbers of samples in clinical studies in order to generate data with sufficient statistical significance.

To achieve high-throughput and reproducible quantification of protein glycosylation in clinical specimens, we developed a method that specifically isolates glycosylated peptides coupled with quantitative analysis using mass spectrometry (MS). In this approach, glycosylated peptides are isolated from complex mixtures with solid-phase extraction of *N*-linked glycoprotein/peptide (SPEG)^{16–17}. Initially, glycoproteins are digested to generate peptides containing glycosylated and non-glycosylated peptides. Next, the *cis*-diol groups of carbohydrates in the glycopeptides are oxidized to aldehydes by periodate. The oxidized carbohydrates are then immobilized on a solid support. The formerly *N*-linked glycosylated peptides are released from the solid phase using PNGase F and subsequently identified with LC-MS/MS. This method simultaneously allows the identification of *N*-linked glycosylated proteins, the site(s) of *N*-linked glycosylation, and the relative quantity of the identified glycopeptides. Moreover, the chemical reaction can be used to selectively oxidize and capture sialic acid-containing glycopeptides using a mild oxidation condition (1 mM periodate and 0°C)¹⁸.

Selected Reaction Monitoring (SRM) using triple quadrupole mass spectrometers coupled with heavy-isotope-labeled-peptide standards is a sensitive and accurate method for high-throughput quantitative analysis of target proteins^{19–24}. In SRM assays, the targeted peptide is selected as a precursor ion in the first quadrupole (Q1), allowed to transfer into the second quadrupole (Q2) and then fragmented in the collision cell. Specific fragment ions generated by Q2 fragmentation are selected in the third quadrupole (Q3) and collected by the detector. The two-step ion selection method, which eliminates most non-targeted mass spectrometry signals, significantly reduces the background noise and, consequently, increases the signals for selected ion transitions.

Prostate-specific antigen (PSA) is the best known diagnostic marker for prostate cancer. However, the poor specificity in patients with PSA concentration between the 2.5–10 ng/mL range (diagnostic gray zone) poses a limitation to its clinical performance^{25–26}. Other forms of PSA, including free-PSA, percent of free-PSA, Pro-PSA, and glycosylated PSA, have been shown to improve the clinical performance of PSA^{26–29}. In this study, we developed SRM assays to monitor glycosylated and sialylated PSA to investigate glycosylation changes of PSA in prostate cancer. Formerly *N*-linked glycosylated and sialylated PSA peptides from prostate cancer and non-cancer tissues were isolated using the enrichment methods described above and quantified with SRM. The results showed that glycosylated, sialylated, and total PSA proteins and their distribution in cancer and non-cancer tissues were not correlated. This suggests that sialylated PSA isoforms are differentially expressed in prostate cancer and non cancer tissues, and thus may be potentially be useful as independent biomarkers for prostate cancer.

EXPERIMENTAL PROCEDURES

Materials and reagents

Tris (2-carboxyethyl) phosphine (TCEP) was purchased from Pierce (Rockford, IL); Sequencing grade endoproteinase Arg-C was from Roche (Penzberg, Germany); peptide-*N*-glycosidase F (PNGase F) was from ProZyme (San Leandro, CA); PSA protein was from Calbiochem (San Diego, CA); heavy-isotope-labeled-peptide standard of PSA glycopeptides was from Cell Signaling Technology (Danvers, MS); Sodium periodate and hydrazide resin were from Bio-Rad (Hercules, CA); C18 and MCX desalting columns were from Waters (Milford, MS); All other chemicals were purchased from Sigma (St. Louis, MO). The HPLC-mass spectrometry (MS) platform, which includes a TSQ Quantum Ultra Triple Stage Quadrupole Mass Spectrometer, Accela High Speed LC system, Hypersil GOLD HPLC Column, and HPLC grade reagents for HPLC-MS analysis were from Thermo Fisher Scientific (Waltham, MA).

Clinical samples

Tissue specimens and clinical information were obtained with informed consent and performed with the approval of the Institutional Review Board of University of Washington. Prostate tissues were obtained from resected glands and the histology of the tissue specimens was assessed by examining adjacent sections. The proteins from cancer and non-cancer tissues were collected using the procedures described in our previous studies^{30–31}. Total PSA concentrations were measured using the Hybritech PSA assay on the Access Immunoassay System (Beckman Coulter, Inc., CA). Pooled serum samples from healthy women spiked with PSA was prepared as described previously³². Different amounts of PSA protein (0 pg, 20 pg, 0.1 ng, 0.2 ng, 1 ng, 2 ng of PSA standard protein) were spiked into 20 μ L of pooled serum samples from healthy women with PSA concentration < 0.01 ng/ml. The final PSA concentrations for each PSA spiked serum samples were measured using the routine clinical PSA assay on the Access Immunoassay System. Sera with different PSA concentrations were prepared in triplicate for extraction of the *N*-linked glycopeptides for MS analysis.

N-linked glycosylated and sialylated peptide isolation

Formerly *N*-linked glycosylated peptides were isolated from tissues using the *N*-linked glycopeptide capture procedure as described previously³². Briefly, serum samples (20 μ L) spiked with different amounts of PSA or tissue proteins (0.2mg) were first digested using 10 μ g of proteinase Arg-C in 100 μ L Arg-C digestion buffer (100mM Tris-HCl, 20mM CaCl₂, 10mM DTT, 1mM EDTA, 40mM Methylamine, adjust pH to 7.6) at 37 °C overnight with gentle shaking. Next, digested peptides were purified with C18 desalting columns. Purified tryptic peptides from tissue samples were separated into two fractions for *N*-linked glycopeptide capture and *N*-linked sialylated glycopeptide capture, respectively. Total *N*-linked glycopeptides from tissue and serum samples were oxidized by 10mM sodium periodate in 50% acetonitrile at room temperature for 1 hour in the dark. *N*-linked sialylated glycopeptides from tissues were oxidized by 1mM sodium periodate in 50% acetonitrile at 4 °C for 15 min in the dark. Oxidized peptides were purified with C18 desalting columns to remove the oxidant and conjugated to hydrazide resin at room temperature for 4 hours in 80% acetonitrile. Unconjugated peptides were then removed by washing the resin three times with 800 μ L of 1.5M NaCl, H₂O, and 100 mM of NH₄HCO₃. *N*-linked glycopeptides or *N*-linked sialylated glycopeptides were released from the resin by 0.5 μ L of PNGase F in 100 mM of NH₄HCO₃ and incubated at 37 °C overnight. After final clean up by MCX desalting columns, the peptides were dried and resuspended in 10 μ L of 0.4% acetic acid solution for mass spectrometry analysis.

Analysis of glycosylated and sialylated PSA using HPLC-TSQ-SRM-MS platform

Ten μL of resuspended glycopeptide solution from 0.1 mg of tissue or 20 μL of serum was used in each LC-MS analysis. Approximately 10 pmole of heavy-isotope-labeled PSA peptide was added to each sample for quantitative detection. All SRM assays were developed on a TSQ Quantum Ultra triple quadrupole mass spectrometer, Surveyor MS pump, Micro Autosampler and an IonMax Source equipped with a low flow metal needle (Thermo Fisher Scientific), flow rate 200 $\mu\text{L}/\text{min}$. Reverse-phase separations were carried out on a 1mm $\times$ 150mm Hypersil Gold 1.9 μm C18 particle. Solvent A was LC-MS grade water with 0.2% (v/v) formic acid, and solvent B was LC-MS grade acetonitrile with 0.2% (v/v) formic acid. The mobile phase B was increased from 5% to 40% in 20 min. The HPLC was plumbed using 1/32 red peek tubing. The instrument divert valve was switched to waste before and after the peptides eluted in order to keep the source free of excess salts and debris. Polytyrosine-1,3,6 calibrant was obtained from CS Bio Company, Menlo Park, CA, product Number CS0272S.

The TSQ Ultra was run in unit resolution with Q1 and Q3 set to 0.7 FWHM. The instrument operating software was Xcalibur 2.0.7 SP1 and TSQ 2.2.0. Cycle times were optimized to ensure a minimum of 12 scans across each peak. The method was run with a cycle time of 1 second.

Heavy-labeled peptides and SRM transitions

Isotopically labeled heavy peptide (incorporating ^{13}C - and ^{15}N -labeled aspartic acid) was synthesized by Cell Signaling Technology (Danvers, MA). Formerly N-linked glycosylated PSA peptide dKSVILLGR (d is labeled aspartic acid for heavy peptide) was used as standard for SRM monitoring. The m/z values were 500.812 for the native peptide $[\text{M}+2\text{H}^+]$ and 503.312 for the heavy peptide $[\text{M}+\text{heavy labeled}+2\text{H}^+]$.

RESULTS

Development of SRM assay for PSA glycopeptide quantification

The selection of fragment ion transitions for the targeted peptide is a critical step for the establishment of a sensitive and reproducible SRM assay. PSA has one N-linked glycosylation site and the deglycosylated glycopeptide, N#KSVILLGR, was used as precursor ion (amino acid N# is the N-linked glycosylation site and converted to D after deglycosylation). To determine the fragment ions of N#KSVILLGR, the synthesized heavy PSA glycopeptide was injected into triple quadrupole MS to generate the entire MS/MS spectrum (Figure 1-A) in order to identify the fragment ions for optimal transitions. Four fragment ions: 249.12 (2b), 345.24 (3y), 670.50(6y), and 757.51(7y) were selected from the MS/MS spectrum. To test the specificity and reproducibility of the four fragment ions in the complex mixture, the same amount of heavy PSA glycopeptides were spiked into pooled sera from healthy women and run multiple times using SRM mode for simultaneous monitoring of the four ion transitions (Figure 1-B). The peak intensity and peak area of each transition was calculated to determine the transitions with the best specificity and reproducibility. Two of four transitions, 670.50 and 757.51, provided the highest specificity and intensity of signal. Therefore, they were selected for establishment of the SRM assay for PSA glycopeptide quantification.

We then determined the sensitivity of the SRM assay for glycosylated PSA in serum by spiking different amounts (20 pg to 1 ng) of standard PSA protein into 20 μL of pooled serum from healthy women donors. The control serum with spiked PSA were analyzed by clinical immunoassay and the PSA concentrations in these samples were 0.50, 1.43, 3.44, 26.87, and 51.43 ng/mL.³² Glycopeptides were isolated from the PSA serum samples in

triplicate, and the same amount of heavy glycopeptide was added into each sample as internal standard. The mixed samples containing native and heavy PSA glycopeptides were subsequently analyzed by SRM (Figure 1-C). Serum from healthy women donors was used as a negative control (PSA protein is not present in female blood). Moreover, since serum represents the most complex biological sample, the assay could potentially be easily applied to other clinical specimens, such as tissue or urine samples. In this study, the assay limit of quantitation (LOQ) (the lowest quantity of PSA glycopeptide that could be distinguished from the background noise ($S/N > 10$)) was 1.43 ng/mL. The low nanogram/milliliter LOQ may facilitate early detection of prostate cancer where serum PSA concentrations are in the range of 4 ng/mL²⁵. Linearity of the assay was excellent with $R^2 = 0.9442$ for a range of 1.43 ng/mL to 51.43 ng/mL. The Coefficient of Variation (CV) was between 5% (for 51.43 ng/mL) to 16.11% (for 1.43 ng/mL), indicating a robust quantitative result for PSA at the clinically relevant range (Figure 2). The LOQ and CV of the SRM assay demonstrate sensitivity and reproducibility in quantitative measurements for glycosylated PSA protein in clinical specimens.

Total PSA protein quantification using clinical immunoassay

Total PSA concentrations in the prostate cancer tissues were measured using the Hybritech PSA assay on the automatic Access Immunoassay System followed by standard operating protocol. Total protein (0.1 μ g) from each prostate cancer and non-cancer tissues were used for the total PSA analysis. The quantitative results represented the total PSA content in each prostate cancer and patient-matched non-cancer tissues (Figure 3-A). The data showed that the total PSA from cancer and non-cancer tissues were not significantly different ($P = 0.8397$) in this sample set.

Glycosylated PSA quantification using isolation of N-linked glycopeptides and SRM analysis

Solid phase extraction of N-linked glycopeptides (SPEG) oxidizes glycan groups, captures glycosylated peptides from complex sample to a solid support, and enzymatically releases deglycosylated glycopeptides. SRM analysis of glycopeptides captured by SPEG at the specific N-linked glycosylation site can be used to measure glycosylated PSA instead of total PSA protein. The isolated formerly N-linked glycopeptides from prostate cancer and patient-matched non-cancer tissues were quantified using heavy-isotope-labeled PSA peptide and the related amounts in each individual were determined (Figure 3-B). The data showed that the relative abundance of glycosylated PSA was not correlated with the total PSA protein level in each tissue sample (Figure 3-A and Figure 3-B) and the total glycosylated PSA from cancer and non-cancer tissues were not significantly different ($P = 0.4859$, Figure 3-B).

Sialylated PSA quantification using sialylated glycopeptide isolation coupled with SRM analysis

In a similar approach to the SPEG method for total N-linked glycosylation, sialylated glycopeptide isolation were applied to enrich sialylated glycopeptides from complex mixtures by modified oxidation conditions specific for glycans containing sialic acid. The formerly N-linked sialylated glycopeptides isolated from each tissue were quantitatively analyzed using the heavy peptide and SRM. The ratio of native to heavy peptide for each tissue sample was used to measure the relative abundance of sialylated PSA in cancer and non-cancer tissues (Figure 3-C). The sialylated PSA did not correlate to total PSA or glycosylated PSA in each sample. However, the sialylated glycopeptide was elevated in cancer tissues compared to non-cancerous tissues ($P = 0.0511$, Figure 3-C).

DISCUSSION

In this study, we developed high-throughput SRM assays for simultaneous analysis of different PSA protein glycosylation isoforms. To our knowledge, this is the first high throughput approach demonstrated for quantification of different protein glycosylations in clinical specimens, which will be a critical tool to investigate the protein glycosylation as potential biomarkers. When we used the developed SRM assays to compare glycosylated and sialylated PSA levels to the total PSA protein amount, we found that the relative abundance of glycosylated PSA, sialylated PSA, and total PSA were not correlated in cancer and non-cancer tissues (Figure 3). The resulting P values between cancer and non-cancer groups from these samples were from 0.8397 for total PSA, 0.4859 for glycosylated PSA, and 0.0511 for sialylated protein, respectively. In our study, sialylated PSA was shown to distinguish cancer from non-cancer groups. Clinical performance of this result for cancer-specific detection will, of course, require verification of with clinical cohorts containing larger numbers of samples. In addition, if the sialylated PSA isoforms from cancer tissues are detected in serum, the specificity of prostate cancer diagnosis in serum may be improved. The results indicated that PSA glycosylation isoforms can be used as additional factor for discriminating cancer and non-cancer tissues. This observation is consistent with results from several clinical studies that identified glycosylation changes between different disease stages^{4, 38–39}.

Glycosylation is an important protein modification associated with disease progression. Identification of altered glycosylation on glycoproteins has increasingly played an important role in clinical research for biomarker discovery. PSA glycosylation patterns as putative biomarkers have been reported by several studies^{33–36}. In these studies, PSA protein glycan structures were identified in clinical samples by MS following exoglycosidase digestion, chromatography or electrophoresis separation or lectin-based technologies^{34, 37}. However, the reported results were inconclusive due to limited sample throughput, reproducibility, and statistical significance. Sialylation, a generic form of glycosylation, has been shown to change in prostate cancer. However, some of the reported results are contradictory and require further evaluation^{34, 38–41}. Therefore, a high-throughput, quantitative assay for PSA glycosylation and sialylation could potentially be useful to investigate the PSA glycosylated isoforms for prostate cancer diagnosis. Our study provides an approach useful for the investigation of N-linked glycosylation and N-linked sialylation of PSA and their clinical utilities for prostate cancer diagnosis. To reveal changes in other glycosylation isoforms, selective isolation of glycopeptides using methods such as lectin or antibody affinity chromatography are likely to provide information on different glycosylation patterns.

Currently, SRM detection is an emerging MS-based method for specific, sensitive, and rapid quantification of targeted proteins. For example, total PSA protein in serum samples has been quantified by SRM coupled to depletion of high-abundance proteins or MSn detection^{32–33}. The assay limit of quantitation (LOQ) in these reports was in the ng/mL range and the quantitative results showed good correlation with existing ELISA immunoassays in sera. Similar results have also been shown in prostate tissues³⁴. In the current study, we have used SRM to develop assays for glycopeptides modified with specific glycans in clinical specimens. The SPEG method allowed the specific enrichment of glycosylated and sialylated glycopeptides from complex samples, thus improving the sensitivity. In addition, the SRM assay increased the detection sensitivity by selectively detecting ion transitions thus reducing interferences. The combination of these techniques resulted in greatly improved signal-to-noise and allowed for high throughput quantitative analysis of different glycosylated isoforms from clinical specimens. Moreover, SRM detection was able to significantly improve the reproducibility of the quantitative analysis. In comparison to a previous study from our laboratory using data-dependent acquisition

(DDA) coupled to MALDI-MS detection, the sensitivity was improved modestly from 3.44ng/mL to 1.43ng/mL, while the %CV was dramatically decreased from 45.01% to 16.11%³². Using the developed SRM assays, we were able to detect PSA glycopeptides in clinical specimens in a specific, sensitive, reproducible, and high-throughput fashion.

In summary, we have established a method for quantitative measurement of specifically glycosylated proteins using glycopeptide extraction coupled to high-throughput SRM-based assay. *N*-linked glycosylated and sialylated PSA isoforms in prostate cancer tissues were monitored in a multiplexed assay and compared to total PSA proteins measured by immunoassay. This provides a new direction for disease-associated glycoproteomic research and may help bridge the gap between biomarker discovery and clinical evaluation of glycosylation isoforms of glycoproteins.

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ABBREVIATIONS

SRM	selected reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
HPLC	high-performance liquid chromatography
SPEG	solid phase extraction of <i>N</i> -linked glycopeptide
PSA	prostate-specific antigen
CV	coefficient of variation
LOQ	limit of quantitation

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1-A

1-B

Figure 1.

The MS-detection of PSA glycopeptide using triple quadrupole mass spectrometry. (A) MS/MS spectrum of PSA glycopeptide dKSVILLGR. (B) SRM spectrum of four fragment ion transitions of PSA glycopeptide dKSVILLGR. (C) Chromatography spectra of selected

fragment ions of native and heavy dKSVILLGR glycopeptides. (d: heavy labeled aspartic acid for heavy peptide, M+5).

Figure 2.
The calibration curve of SRM detection of glycosylated PSA in human serum.

A

B

C

Figure 3.

Analysis of total protein, *N*-linked glycosylated PSA, and *N*-linked sialylated PSA in prostate cancer and non-cancer tissues.